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Study of the retention behavior in zwitterionic hydrophilic interaction chromatography of isomeric hydroxy- and aminobenzoic acids

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ABSTRACT

The retention behavior of fifteen isomeric hydroxy- and aminobenzoic acids in zwitterionic hydrophilic interaction chromatography was studied using a sulfobetaine phase (ZIC-HILIC). By an inspection of their molecular structures, the retention was related to the number, the position and hydrogen bond properties of the functional groups. The effect of the chromatographic conditions was analyzed in order to investigate the retention mechanism of the stationary phase. The increased retention observed for negative charged compounds when the mobile phase pH decreased was ascribed to a diminishing of the electrostatic repulsion with the underivatized silanol groups. Also the salt buffer concentration in the mobile was proved to have a great influence in the modulation of the electrostatic interactions. However, the retention behavior of the benzoic acids was not described by conventional ion-exchange models. Subsequently, a systematical analysis of partition, adsorption, and hydrophilic chromatographic models was presented. The results from the fittings indicated that partition processes govern mainly the ZIC-HILIC separation, but also adsorption processes via hydrogen bonds occurred for hydrogen donor analytes. Finally, the influence of the chromatographic conditions on the water enriched layer in which partition takes place has been evaluated by the elution behavior of toluene.

1. Introduction

Hydrophilic interaction liquid chromatography (HILIC) is a quite recent but well established technique, popular for its complementary selectivity to reversed phase (RP) liquid chromatography [1]. This characteristic makes HILIC suitable for the separation and the analysis of hydrophilic and polar compounds, generally poor retained by the widespread RP liquid chromatography. In the last twenty years HILIC separation has found application in many fields of research, from peptides, carbohydrates and nucleic acids [2–4], to pharmaceutical, biomolecular, food, metabolic, and pollutant compounds [5–9].

The HILIC separation mode is based on the combination of a polar stationary phase with a less polar aprotic organic-rich mobile phase, which is in most cases an aqueous-acetonitrile mixture where the water is the stronger eluting solvent [2].

The retention in HILIC is discussed as a mixed-mode mechanism. Partitioning of the analyte between the organic-rich mobile phase and the water enriched layer partially immobilized on the stationary phase is regarded as the main retention mechanism [2]. However, also other important interactions as hydrogen bonds, electrostatic interactions, and dipole–dipole interactions contribute to the HILIC separation [10–13].

Any polar stationary phase which can retain water could be used in HILIC mode. Although the first applications were simply based on nonbonded silica, the increasing interest in polar compound analysis has recently prompted the development of different stationary phases, suitable for a wide range of analyses [14]. Clearly, the retention mechanism of the analyte depends on the characteristic of the stationary phase and on the specific interactions that can drive the separation [10,15,16].

Among all the commercially available columns, an interesting and very common type of stationary phases is that with zwitterionic ligands bonded on silica, as the ZIC-HILIC introduced by SeQuant/Merck. As recently reviewed by Nesterenko et al. [17], zwitterionic stationary phases contain equal amounts of oppositely charged groups not sensitive to pH, bonded in close proximity of the surface. The ZIC-HILIC phase, for example, is in a permanently charged state, due to the quaternary ammonium and sulfonate groups in the sulfobetaine ligand. This phase has been proved to achieve with both positive and negative charged solutes ionic and weak electrostatic interactions, in addition to hydrophilic interactions. For this reason the sulfobetaine phase has found several applications in the analysis of acid, basic and zwitterionic compounds [16,18,19]. Even if the retention mechanism in zwitterionic hydrophilic interaction chromatography is the subject of many

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papers [16,20], a systematical study of the nature of the retention mechanism, as recently reported for different stationary phases [10,21,22], is still lacking.

Aim of this study is to provide further insight into the retention mechanism of a ZIC-HILIC phase and into the parameters that affect it. Fifteen simple polar isomeric hydroxy- and aminobenzoic acids were selected on the basis of their similarities in structure, polarity and charged state. Their retention behavior was considered under several chromatographic conditions. Different retention models, developed for partition, adsorption, and hydrophilic chromatography, were applied, in order to investigate the nature of the retention mechanism. Finally, the simultaneous influence of ACN content and salt concentration in the mobile phase on the thickness of the water enriched layer on the sulfobetaine phase was estimated based on the elution behavior of toluene.

2. Experimental

2.1. Chemicals and materials

Acetonitrile HiPerSolv Chromanorm was purchased from BDH (Poole, UK). Water LC–MS Chromasolv was bought from Fluka (Buchs, Switzerland). Ammonia solution (32%) and formic acid were obtained from AppliChem (Darmstadt, Germany). Toluene, ammonium acetate, 3-hydroxybenzoic acid (3-HB), 2,3-dihydroxybenzoic acid (2,3-DHB), 3,5-dihydroxybenzoic acid (3,5-DHB), 3-amino-4-hydroxybenzoic acid (3,4-AHB), 3,4-diaminobenzoic acid (3,4-DAB), 4-hydroxy-3,5-dimethoxybenzoic acid (syringic acid, SYR), 4-aminobenzoic acid (4-AB), and 2-aminobenzoic acid (2-AB) were purchased from Sigma–Aldrich (Seelze, Germany). 4-Hydroxybenzoic acid (4-HB) was purchased from Merck (Darmstadt, Germany). 2-Hydroxybenzoic acid (2-HB), 2,4-dihydroxybenzoic acid (2,4-DHB), 2,5-dihydroxybenzoic acid (2,5-DHB), 3,4-dihydroxybenzoic acid (3,4-DHB), 3,4,5-trihydroxybenzoic acid (3,4,5-THB), and 4-hydroxy-3-methoxybenzoic acid (vanillic acid, VAN) were provided by Prof. Treutter (TU München, Germany). All chemicals were of analytical-reagent grade.

2.2. Standards

Stock standard solutions of each analyte were prepared at a concentration of 500 mg/L, dissolving the adequate amount of the compound in a mixture of water/ACN (1:1, v/v). All stock solutions were stored at −20 °C. Working standard solutions were obtained from the corresponding stock solutions by dilution (1:100) in mobile phase with respect to the initial conditions. Standard solution of toluene was obtained diluting the appropriate volume in 100% ACN.

2.3. Instrumentation and chromatographic conditions

Chromatographic analyses were conducted on a Knauer PLATINblue UHPLC system (Berlin, Germany), equipped with a cooled autosampler, a binary pump, an on-line degasser, a mixing chamber, a column oven maintained at 20 °C, and a photo diode array detector. ChromGate 3.3.1 software (Knauer) was used for UHPLC-UV data acquisition and control.

The chromatographic analyses were performed with a ZIC®-HILIC column (150 × 2.1, 3.5 μm, 200 Å) (Merck Sequant, Umeå, Sweden). The mobile phase consisted of a mixture of acetonitrile/water (95:5, v/v; solvent A) at various ammonium acetate concentration and pH values and water (solvent B) containing ammonium acetate at concentration equal to those in solvent A. The pH of solvent B was adjusted with ammonia or with formic acid, and the same volume was added to the solvent A too [21].

The final eluent was prepared by mixing appropriate volumes of the two solvents and was delivered isocratically at flow rate of 0.4 mL/min. The compounds were injected one-at-a-time.

The hold-up time (t_0 = 1.10 ± 0.02 min) for calculating retention factors was determined in five separate experiments using the retention time (baseline disturbance) of 2 μL water/ACN (1:1, v/v) injection.

2.4. Data analysis

The pK_a values measured in water were obtained from literature [23–25]. MarvinSketch 5.5.0.1 software (ChemAxon, Budapest, Hungary) was used to calculate the pK_{a2} and pK_b values of the analytes. Microsoft Excel 2007, GLE 4.2, and Mathematica 7.0.1.0 (Wolfram Research Inc., Champaign, IL) were used for the determination of the parameters of Eqs. (1)–(4), statistical data analysis, and plotting functions.

Ten data points acquired in the ACN range 80–95% were used for the evaluation of the retention models. For 3,4-DHB and 3,5-DHB eight data points in the ACN range 80–92% were considered, while for 3,4,5-THB seven data points in the ACN range 80–90%. 2-HB was not included in this analysis, as it showed retention just in a small range of ACN content (i.e. 87–95% (v/v)).

3. Results and discussion

3.1. Effect of acetonitrile content in the mobile phase

The effect of the ACN fraction in the mobile phase on the retention of fifteen isomeric hydroxy- and aminobenzoic acids was studied in the range 80–95% (v/v), at a constant ammonium acetate buffer concentration of 10 mM. The aqueous portion was adjusted to pH 7.0 with an ammonium hydroxide solution, which was added at the same final concentration to the organic portion too. Fig. 1 shows the variation of the retention factor (k') of the analytes as a function of the ACN percentage in the mobile phase. All the benzoic acids exhibited typical HILIC behavior of increasing retention with increasing ACN content. Experiments at lower concentrations of ACN were not performed, as most of the compounds were poorly retained at ACN content below 80% (v/v).

Despite the high content of organic solvent in the mobile phase, the ionization state of the analytes was not different from that in pure aqueous solution (pK_a, pK_{a2} and pK_b values are shown in Table 1). This was experimentally supported by their retention behavior at different ionic strengths and pHs of the mobile phase,

Table 1
Physical properties of hydroxy- and aminobenzoic acids.

Compound	pK_a [23–25]	p$K_{a2\,(OH)}$ [a] or p$K_{b\,(NH2)}$ [a]
2-Hydroxybenzoic acid (2-HB)	2.98	13.23
3-Hydroxybenzoic acid (3-HB)	4.08	9.55
4-Hydroxybenzoic acid (4-HB)	4.58	9.67
2,3-Dihydroxybenzoic acid (2,3-DHB)	2.98	9.64
2,4-Dihydroxybenzoic acid (2,4-DHB)	3.29	9.81
2,5-Dihydroxybenzoic acid (2,5-DHB)	2.97	10.02
3,4-Dihydroxybenzoic acid (3,4-DHB)	4.49	9.41
3,5-Dihydroxybenzoic acid (3,5-DHB)	4.04	9.29
3,4,5-Trihydroxybenzoic acid (3,4,5-THB)	4.42	9.04
4-Hydroxy-3-methoxybenzoic acid (vanillic acid, VAN)	4.52	10.14
4-Hydroxy-3,5-dimethoxybenzoic acid (syringic acid, SYR)	4.21	9.55
2-Aminobenzoic acid (2-AB)	2.09	1.95
4-Aminobenzoic acid (4-AB)	2.41	2.69
3-Amino-4-hydroxybenzoic acid (3,4-AHB)	4.73	3.38
3,4-Diaminobenzoic acid (3,4-DAB)	4.84	3.25

[a] Values calculated with MarvinSketch 5.5.0.1 software.

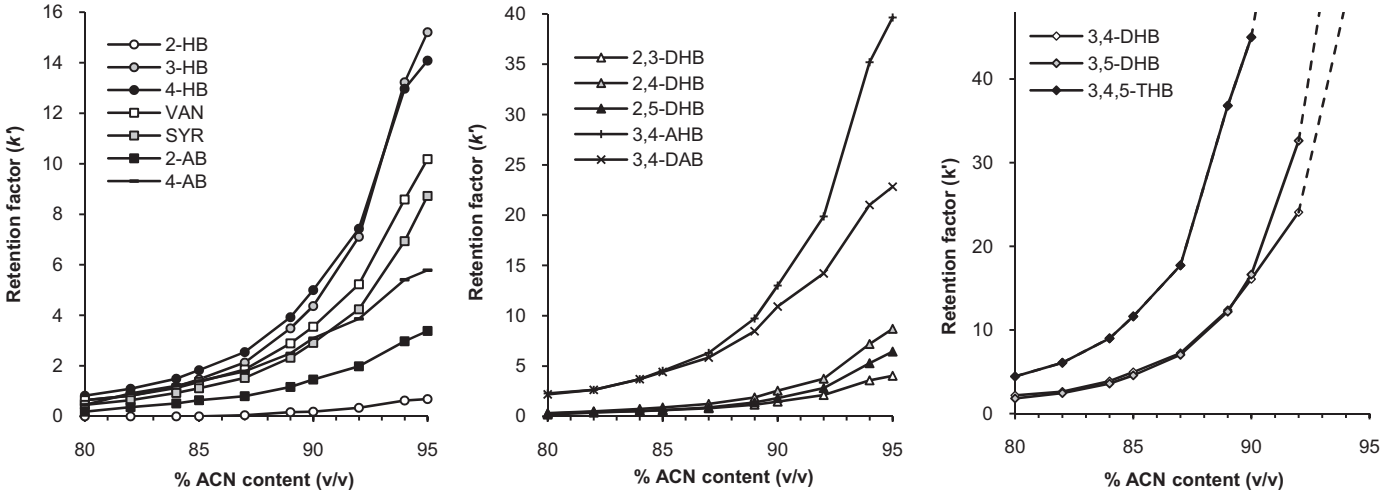

Fig. 1. Effect of ACN content on the retention factor (k') of hydroxy- and aminobenzoic acids. Mobile phase: ACN/water at various percentages; ammonium acetate, 10 mM; pH 7.0; column temperature, 20 °C; flow rate, 0.4 mL/min.

as later discussed. Therefore, all the benzoic acids were in negative charged state, due reasonably to the deprotonation of carboxylic functionality, while the hydroxy and the amino groups were in neutral state.

The elution order of the benzoic acids appeared to be related to the position, number, and type (—OH or —NH_2) of the polar functional groups.

As regard to the effect of the position of the functional groups on the retention behavior, all the hydroxybenzoic acids with a functional group in *ortho* position to the carboxyl group resulted poorly retained. In contrast, the hydroxybenzoic acids with the *ortho* position free were more retained, independently of the number of the other hydroxyl groups. The same behavior was observed for the aminobenzoic acids. The weaker retention of the *ortho*-hydroxy- or *ortho*-aminobenzoic acids was ascribed to the presence of an intramolecular hydrogen bonding of the *ortho* group with the carboxylate anion that prevents the latter to establish intermolecular interaction with the column [26]. This effect was more evident for 2-HB than for 2-AB, indeed the hydroxyl group is a hydrogen donor functionality that can interact strongly with the carboxylate group.

About the effect of the number of functional groups, the elution orders 4-HB (one —OH group) < 3,4-DHB (two —OH) < 3,4,5-THB (three —OH), and 4-AB (one —NH_2 group) < 3,4-DAB (two —NH_2) were observed. An increase of the retention factor was associated to the increasing number of the polar functional groups [27].

Finally, the influence on the retention of functional group type (—OH or —NH_2) was considered. In order to investigate the difference between hydrogen donor (—OH) and hydrogen acceptor (—NH_2) functionalities, the retention behavior of structural related hydroxy- and aminobenzoic acids was compared. Both hydroxyl and amino groups can exhibit medium strength hydrogen bonding [21,28]. The retention orders 4-AB < 4-HB, and 3,4-DAB (two —NH_2) < 3,4-AHB (one —OH, one —NH_2) < 3,4-DHB (two —OH) indicated an increase of the retention factor substituting each amino group with a hydroxyl group. To further inspect the stronger retention of the compounds with hydrogen donor functionalities, the $\log k'$ values were plotted against the pK_{a2} values for the hydroxybenzoic acids and the pK_b values for the aminobenzoic acids (Supplementary Material, Fig. S1). The data showed that the retention factor increased with the decrease of pK_{a2} for the hydroxybenzoic acids, and with the increase of pK_b for the aminobenzoic acids, suggesting a role of the hydrogen bond properties of the analytes in the retention mechanism.

The stronger retention of the hydrogen donor analytes may be attributed to the interaction with the hydrogen acceptor sulfonate group of the zwitterionic phase [29].

Hydrogen bonds with the stationary phase might be disturbed when the hydroxyl group is sterically hindered. To consider this hypothesis, the retention behavior of 4-HB was compared to that of VAN and SYR, differing from the 4-HB for the presence of one and two methoxy groups respectively, in *ortho*-position to the hydroxyl group. The elution order 4-HB > VAN > SYR was observed in all the studied ACN range, confirming that the steric hindrance of the hydroxyl group decreases the interaction with the stationary phase. The decrease in the retention caused by the increasing number of methoxy groups may be also due to a hydrophobicity increase of the solutes.

3.2. Effect of mobile phase pH

The sulfobetaine stationary phase in ZIC-HILIC can be used in the range of pH 3–8 [30]. The effect of the mobile phase pH on the retention behavior of the benzoic acids was investigated at pH 3.0 and 7.0 at a constant ammonium acetate concentration of 10 mM and at ACN/water content of 90/10 (v/v). The pH of the aqueous solutions was adjusted with formic acid or ammonium hydroxide respectively, and the same volumes were added to the organic portion too [21]. Therefore, the hydrophilic interaction and the ionic interaction were kept constant, and the variation of the retention mainly depended on the variation of the charge state of both the analytes and the stationary phase. (The retention time of the compounds at pH 3.0 and pH 7.0 are shown in Fig. S2 in Supplementary Material).

All the benzoic acids with pK_a > 4 displayed a decrease in the retention time between 60 and 90% as the mobile phase pH changed from 7.0 to 3.0.

This effect is simply explained by the benzoic acids protonation that brings them in neutral form. Generally, charged solutes are more hydrophilic than their neutral form and thus more retained in HILIC mode [15]. On the other hand, at pH 3.0 the amino group of the aminobenzoic acids (pK_b 2–3) is expected to be partially protonated, enhancing their hydrophilic character and allowing attractive interaction with the sulfonate group of the sulfobetaine phase. However, this effect was not observed.

The benzoic acids with p$K_a \sim 3$ displayed an opposite behavior. For 2-HB, 2,3-DHB and 2,5-DHB an increase of the retention time of 10–30% was observed. At pH 3.0 these compounds should partially be in charged state, so no variation or at most a decrease

of the retention would be expected, as a result of the decrease of their hydrophilicity. ZIC-HILIC column contains a silica-based phase, where the sulfobetaine groups are in a permanent zwitterionic state not affected by the pH variation [17]. However, the mobile phase pH can change the charge state of the residual silanol groups, whose concentration and accessibility are unknown [18]. The silanol groups at pH 7.0 are deprotonated, contributing to impart a net negative charge to the stationary phase, in addition to the sulfonate group of the sulfobetaine ligands. As the mobile phase pH decreases to 3.0, the amount of charged silanols decreases too, diminishing the electrostatic repulsion with the negative charged analytes. Therefore, the benzoic acids that at pH 3.0 were still in negative charged state were less affected by the electrostatic repulsion with the residual silanols, increasing their retention. 2,4-DHB (pK_a 3.29), that at pH 3.0 should be in equilibrium between the charged and the neutral form, presented a modest decrease of the retention. This behavior can be understood considering a balance between the negative effect of the decreased hydrophilicity of the solute by carboxylate protonation and the positive effect of the decreased electrostatic repulsion with the residual silanols.

3.3. Effect of salt concentration in the mobile phase

Both the quaternary ammonium (positively charged) and the sulfonate group (negatively charged) of sulfobetaine ligand of the ZIC-HILIC stationary phase may be responsible of electrostatic interaction with charged analytes [15,19,20]. The effect of the ammonium acetate concentration on the retention was investigated in the range 1–20 mM. The buffer concentration refers to the final concentration in the mobile phase. All the analyses were performed with a mobile phase consisting of ACN/water 90/10 (v/v) at pH 7.0. As shown in Fig. 2, the retention factor for all the benzoic acids increased with the increasing ionic strength.

This trend is consistent with previous observations, according to which, even if the surface charge of the sulfobetaine phase should be zero, the sulfonate group at the distal end of the phase gives to the ZIC-HILIC material a significant net negative charge [19,20,30]. The buffer salt surrounds the charged groups, diminishing the electrostatic interaction. The observed increase of the retention is related to a decrease of the electrostatic repulsion of anionic acids with the sulfonate group. It has to be pointed out that even if the salt concentration had a greater influence on the benzoic acids without any functional group in *ortho* position, no variation of the elution order occurred. The good retention observed for most compounds even at low buffer concentration, when the electrostatic repulsion with the stationary phase is higher may be caused by hydrophilic interactions achievable owing to a proper orientation of the analytes. This combination has been described by Alpert and is known as ERLIC (electrostatic repulsion–hydrophilic interaction chromatography) [19]. In accordance with this view, the retention factor k' plotted against the inverse of counter-ion concentration in the mobile phase did not show a linear trend, as should be for a conventional ion-exchange mechanism (Supplementary Material, Fig. S3). Previous works have already reported a non-linear trend [18,20]. In particular, McCalley has recently observed a good accordance with a second order polynomial expression for basic compounds on ZIC-HILIC [20]. However, the experimental data for benzoic acids were not well described by the same expression.

3.4. Comparison of different retention models

Some common retention models were applied to investigate the nature of the retention mechanism of the benzoic acids in zwitterionic hydrophilic interaction chromatography [10,22].

Table 2
Squared correlation coefficients (R^2) for partition (Eq. (1)) and adsorption (Eq. (2)) models for hydroxy- and aminobenzoic acids.

Compound	R^2	
	Eq. (1)	Eq. (2)
3-HB	0.9904	0.9860
4-HB	0.9931	0.9812
2,3-DHB	0.9863	0.9835
2,4-DHB	0.9931	0.9836
2,5-DHB	0.9934	0.9726
3,4-DHB	0.9823	0.9952
3,5-DHB	0.9837	0.9997
3,4,5-THB	0.9829	0.9969
VAN	0.9934	0.9842
SYR	0.9964	0.9815
2-AB	0.9894	0.9375
4-AB	0.9820	0.9173
3,4-AHB	0.9854	0.9864
3,4-DAB	0.9958	0.9677

Initially, the basic equations describing pure partitioning and adsorptive interactions were considered. RP liquid chromatography has its roots in liquid–liquid chromatography, where the retention is controlled by partitioning. The empirical equation established for partitioning in RP liquid chromatography is [10,31]:

$$\log k' = \log k'_A - S\varphi \quad (1)$$

where φ is the volume fraction of the stronger component of a binary mobile phase, and k'_A is the retention factor of the solute for the weaker eluent only as mobile phase. In the case of HILIC, φ is the volume fraction of water in the mobile phase [22].

For normal phase chromatographic systems, where the retention is based on surface adsorption, the relationship between the retention and the volume fraction of the stronger solvent in the eluent is well described by the following equation [10,32,33]:

$$\log k' = \log k'_B - n \log \varphi \quad (2)$$

where k'_B is the retention factor with pure stronger solvent as eluent.

In several HILIC studies plots of $\log k'$ vs. φ and $\log \varphi$, as shown in Figs. 3 and 4 for the benzoic acids, were used to have an indication on whether partitioning or adsorption was the dominating retention mechanism [10].

The regression results based on Eqs. (1) and (2) are shown in Table 2. The linear regression based on Eq. (2) provided correlation coefficients above 0.995 for 3,4-DHB, 3,5-DHB, and 3,4,5-THB. Considering the mole fraction of water, instead of its volume fraction [22,34], as described by the original Snyder model, lower correlation coefficients were found for all the compounds. In all the other cases Eq. (1) provided better fittings. For the compounds that presented correlation coefficients from Eq. (1) higher than Eq. (2) the residual at φ was evaluated. This is defined as the difference between the experimental value of $\log k'$ and the value estimated from Eq. (1) with the parameters obtained from the regression. If Eq. (1) described well the variability of $\log k'$ with φ, the residuals would be randomly distributed around zero. Instead, the plot of the residuals vs. φ displayed for all compounds except for the aminobenzoic acids (2-AB, 4-AB, 3,4-DAB) a peculiar U-shape, indication of a $\log k'$ dependency on φ beside that described by Eq. (1) (for plots of the residuals of selected compounds, see Supplementary Material, Fig. S4).

These observations pointed out that the retention of most benzoic acids on the sulfobetaine column at high content of organic solvent in the mobile phase was completely described neither by a pure partition nor by a pure surface adsorption model.

In order to more accurately described dependency of $\log k'$ on φ two retention models with three regression parameters were

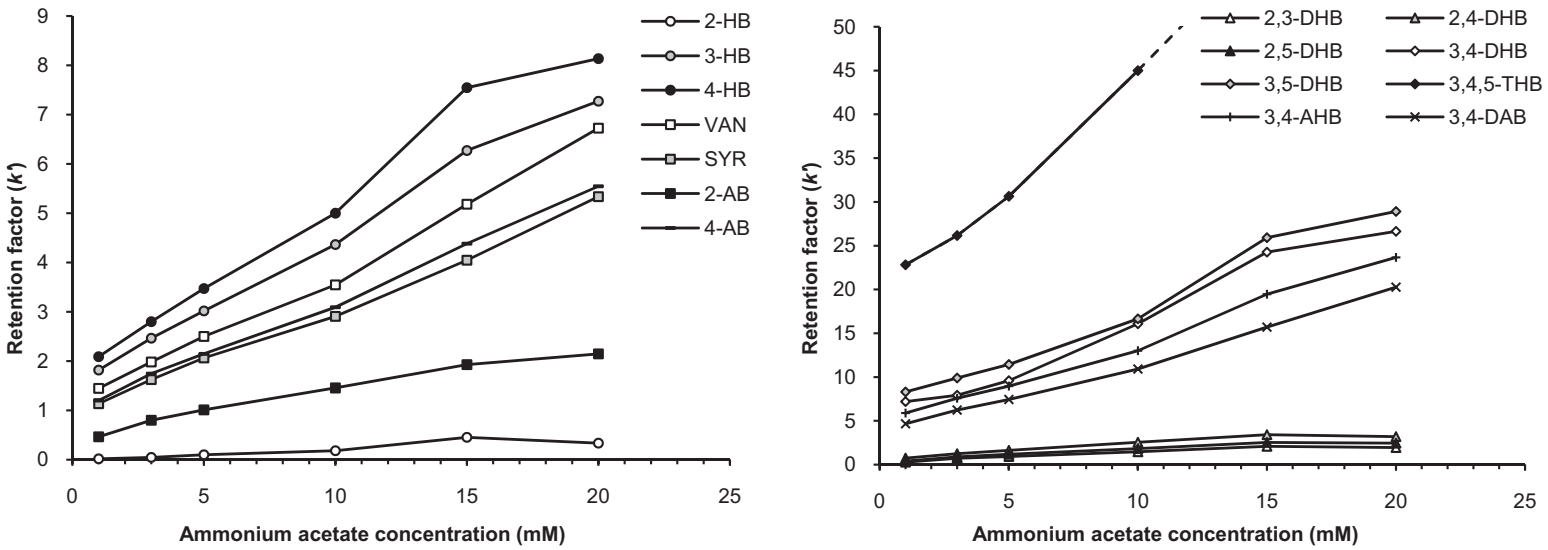

Fig. 2. Effect of salt concentration on the retention factor (k') of hydroxy- and aminobenzoic acids. Mobile phase: ACN/water 90/10 (v/v); ammonium acetate at various concentrations; pH 7.0; column temperature, 20 °C; flow rate, 0.4 mL/min.

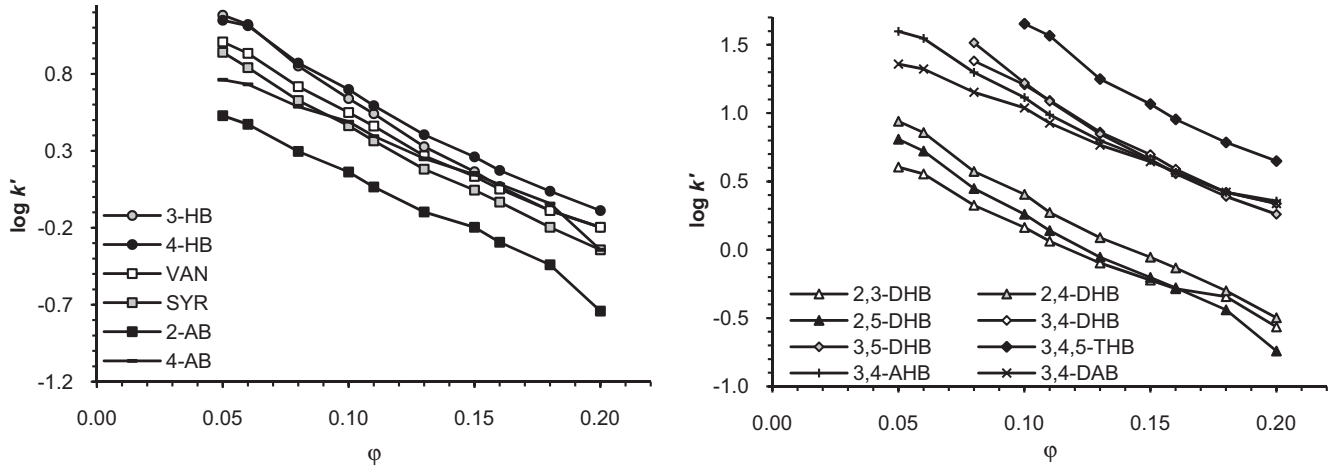

Fig. 3. Plots of log k' of hydroxy- and aminobenzoic acids vs. the volume fraction of water (φ) in the mobile phase. Conditions: ACN/water at various percentages; ammonium acetate, 10 mM; pH 7.0; column temperature, 20 °C; flow rate, 0.4 mL/min.

additionally applied. A partition-like mechanism in RP liquid chromatography, besides Eq. (1), can be also described by the equation [10,22,35]:

$$\log k' = A\varphi^2 + B\varphi + C \quad (3)$$

A third model, recently introduced by Liang and co-workers for HILIC systems was also evaluated. This includes simultaneously the interaction of the solute both with the solvent and with the stationary phase [14,21,22]:

$$\ln k' = a + b\ln\varphi + c\varphi \quad (4)$$

The regression parameters of Eqs. (3) and (4), along with their standard errors and p-values, and the squared correlation coefficients are shown in Tables 3 and 4 respectively.

In the most cases both models well described the retention behavior of the analytes, providing correlation coefficients above 0.99, and in all cases improved with respect to the regressions based on Eq. (1) or (2). Yet, the presence of three regression parameters instead of two could be responsible of the observed improvement. To judge the necessity of one additional term in the regression, the hypothesis that the coefficients A and B of Eq. (3) and b and c of Eq. (4) were different from zero was tested by the analysis of their p-values. The aminobenzoic acids (2-AB, 4-AB, 3,4-DAB) showed A coefficients and b coefficients with high p-values above 0.08. When A and b are equal to zero, Eqs. (3) and (4) reduce to Eq. (1). In these cases the use of the multiparametric models was not justified, and the retention mechanism of aminobenzoic acid on sulfobetaine column was well described by a linear partition model. In contrast, the hydroxybenzoic acids 3,4-DHB, 3,5-DHB, and 3,4,5-THB had c coefficients of Eq. (4) with high p-values above 0.2. When c is equal to zero Eq. (4) reduces to Eq. (2). For these hydroxybenzoic acids the data were well described both by Eqs. (2) and (3).

Nevertheless, as general trend Eq. (3) fitted better than Eq. (4) the retention behavior of the amino benzoic acids, VAN, and SYR, while Eq. (4) that of the hydroxybenzoic acids.

Recent works have shown that at high ACN content in the mobile phase the water enriched layer is relatively low and direct interaction between the solutes and the stationary phase are favored [21,36]. Sulfobetaine polymers are known to retain water strongly [37]. So, they are considered a near ideal phase for liquid–liquid partitioning [10]. In accordance with this view, the retention behavior of most benzoic acids was better described by pure partition models (Eqs. (1) and (3)). The good fit of the data also with Eq. (4) suggested the contribution to the retention mechanism of adsorption-like processes, besides hydrophilic partitioning, at least for the hydroxybenzoic acids. As previously observed, the retention mechanism in HILIC is dependent on both the solute characteristics and the stationary phase [10,15]. The different behavior of similar compounds on the same stationary phase, under the same chromatographic conditions were ascribed to the structural feature of the analytes, where the hydrogen-donor properties of the hydroxybenzoic acids may contribute to the superimposition of adsorption-like processes

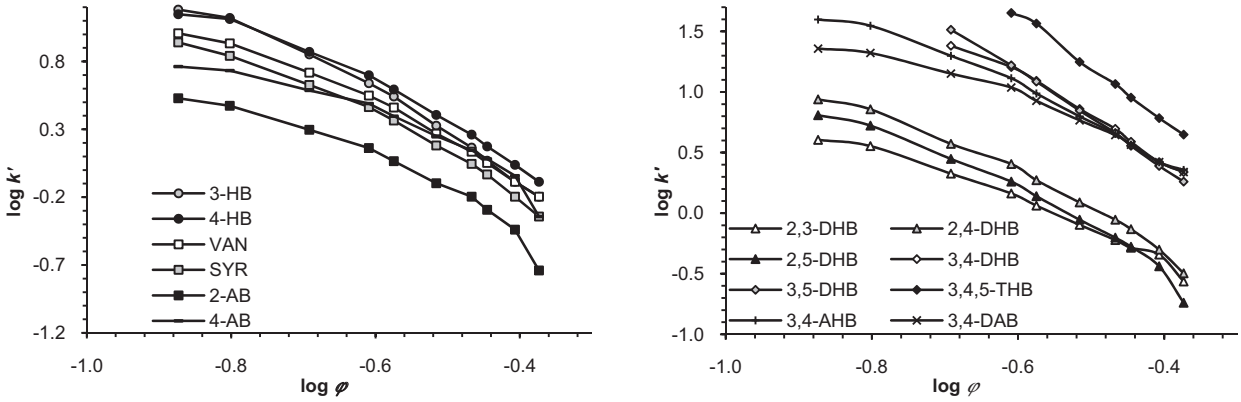

Fig. 4. Plots of log k' of hydroxy- and aminobenzoic acids vs. the logarithm of volume fraction of water ($\log\varphi$) in the mobile phase. Conditions: ACN/water at various percentages; ammonium acetate, 10 mM; pH 7.0; column temperature, 20 °C; flow rate, 0.4 mL/min.

Table 3
Regression data of Eq. (3) for hydroxy- and aminobenzoic acids.

Compound	$C \pm$ standard error	$B \pm$ standard error (p-value)	$A \pm$ standard error (p-value)	R^2
3-HB	1.89 ± 0.05	−14.62 ± 0.75 (<0.001)	20.56 ± 3.01 (<0.001)	0.9987
4-HB	1.75 ± 0.05	−12.09 ± 0.80 (<0.001)	14.36 ± 3.19 (0.003)	0.9982
2,3-DHB	1.16 ± 0.07	−11.54 ± 1.33 (<0.001)	15.74 ± 5.34 (0.02)	0.9939
2,4-DHB	1.56 ± 0.06	−13.10 ± 1.04 (<0.001)	14.79 ± 4.16 (0.01)	0.9975
2,5-DHB	1.37 ± 0.10	−11.66 ± 1.65 (<0.001)	7.20 ± 2.31 (0.02)	0.9943
3,4-DHB	2.52 ± 0.14	−15.72 ± 2.03 (<0.001)	23.60 ± 7.21 (0.02)	0.9963
3,5-DHB	2.95 ± 0.04	−21.10 ± 0.66 (<0.001)	38.26 ± 2.33 (<0.001)	0.9997
3,4,5-THB	3.54 ± 0.20	−22.95 ± 2.81 (0.001)	42.41 ± 9.42 (0.01)	0.9972
VAN	1.58 ± 0.03	−11.89 ± 0.48 (<0.001)	14.85 ± 1.91 (<0.001)	0.9993
SYR	1.47 ± 0.03	−11.26 ± 0.43 (<0.001)	11.07 ± 1.73 (<0.001)	0.9994
2-AB	0.82 ± 0.09	−5.38 ± 1.52 (0.01)	−10.62 ± 6.12 (0.13)	0.9926
4-AB	0.96 ± 0.08	−3.25 ± 0.48 (0.02)	−14.95 ± 3.51 (0.08)	0.9903
3,4-AHB	2.33 ± 0.06	−14.39 ± 0.98 (<0.001)	22.73 ± 3.97 (<0.001)	0.9974
3,4-DAB	1.81 ± 0.05	−8.48 ± 0.90(<0.001)	5.34 ± 3.59 (0.18)	0.9968

F-statistic ranged between 359 and 8394; *Significance* of $F < 7.9 \times 10^{-6}$. The mean of the residuals was $< 7.6 \times 10^{-16}$, with standard deviation between 0.017 and 0.071.

to the hydrophilic partitioning. This interpretation is coherent with the properties of the sulfobetaine phase, where the hydrogen-acceptor sulfonate group can interact with hydrogen-donor solutes. On the contrary, the quaternary ammonium group has no possibility of interacting via hydrogen-bond [29].

3.5. Hydration of the stationary phase

The thickness of the water enriched layer on the surface of a stationary phase plays an important role in the modulation of the retention characteristics of the phase. In addition to the water content in the mobile phase, also the salt concentration has found to have an effect, beside that on the electrostatic interactions. In this regard, several examples of increasing retention time with increasing salt concentration are described in literature. This suggests that the salt might increase the volume of the immobilized aqueous layer on the stationary phase, with ensuing promotion of hydrophilic interaction [15,16]. The presence of the immobilized water layer into which partitioning could be occurring has been recently assessed [38,39]. The effect of the salt is more evident at high percentage of organic solvent in the eluent, because this causes salt to partition preferentially into the water layer. However, until now just few investigations about the simultaneous effect of different chromatographic parameters on the water layer have been conducted. A recent study carried out with a design of experiment approach has indicated that both ACN content and salt concentration in the mobile phase have a significant effect on the retention of organic acids on the sulfobetaine phase [39]. This study has also revealed that the variation of column temperature has a less impact on the retention. Therefore, the temperature effect was no further investigated in the present work.

Previous researches showed a close relation between toluene retention behavior and the thickness of the water layer [21,36]. The retention of toluene at high content of organic solvent in the mobile phase was explained considering that in this condition the stagnant layer is not purely aqueous and this permits toluene partitioning [39]. In order to provide some insight into the simultaneous influence on the water layer of salt and ACN content in the mobile phase, toluene was injected at different salt concentrations in the range 0–15 mM and ACN contents between 80 and 95% (v/v). Sixteen data points were acquired.

The contour plot is reported in Fig. 5, showing that toluene elution time decreases with decreasing ACN content or increasing salt concentration (for plots of toluene elution time vs. ACN content or salt concentration, see Supplementary Material, Figs. S5 and S6). Therefore, both parameters influence the thickness of water layer. Each line represents the ACN-salt contents in the mobile phase for which the retention time of toluene is constant, corresponding to an equal volume of the aqueous layer. So, for example, the volume of the layer created by a mobile phase of ACN/water 88/12 (v/v) in absence of salt is comparable to the layer produced by a mobile phase of ACN/water 95/5 (v/v) in presence of a salt concentration of 15 mM.

The greater change on the retention was observed for ACN content variation, even if the salt effect was also relevant. The two effects were not independent, indeed, for example, that of the salt was stronger at high ACN content, in accordance with the literature [39].

Table 4
Regression data of Eq. (4) for hydroxy- and aminobenzoic acids.

Compound	$a \pm$ standard error	$b \pm$ standard error (p-value)	$c \pm$ standard error (p-value)	R^2
3-HB	0.25 ± 0.80	−1.06 ± 0.24 (0.003)	−12.42 ± 2.21 (<0.001)	0.9995
4-HB	1.22 ± 0.74	−0.72 ± 0.22 (0.01)	−13.17 ± 2.06 (<0.001)	0.9972
2,3-DHB	−0.74 ± 0.97	−0.89 ± 0.29 (0.02)	−9.59 ± 2.71(0.01)	0.9941
2,4-DHB	0.24 ± 0.68	−0.88 ± 0.20 (0.004)	−13.81 ± 1.88(<0.001)	0.9982
2,5-DHB	1.18 ± 1.14	−0.53 ± 0.17 (0.02)	−17.96 ± 3.19 (<0.001)	0.9950
3,4-DHB	−0.33 ± 2.15	−1.68 ± 0.48 (0.01)	−8.37 ± 1.57 (0.18)	0.9973
3,5-DHB	−4.21 ± 0.58	−3.08 ± 0.19 (<0.001)	−0.81 ± 1.45 (0.60)	0.9998
3,4,5-THB	−6.02 ± 2.90	−4.19 ± 0.98 (0.01)	4.59 ± 6.82 (0.54)	0.9984
VAN	0.64 ± 0.52	−0.78 ± 0.16 (0.002)	−11.95 ± 1.45 (<0.001)	0.9985
SYR	0.97 ± 0.28	−0.63 ± 0.08 (<0.001)	−13.90 ± 0.77 (<0.001)	0.9996
2-AB	3.70 ± 1.23	0.45 ± 0.37 (0.25)	−22.51 ± 1.41 (<0.001)	0.9913
4-AB	5.04 ± 1.24	0.72 ± 0.38 (0.09)	−22.51 ± 3.46 (<0.001)	0.9900
3,4-AHB	0.86 ± 1.03	−1.13 ± 0.31 (0.008)	−9.92 ± 2.86 (0.010)	0.9950
3,4-DAB	3.30 ± 0.71	−0.20 ± 0.22(0.38)	−14.62 ± 2.01 (<0.001)	0.9963

F-statistic ranged between 401 and 9030; *Significance* of $F < 6.4 \times 10^{-6}$. The mean of the residuals was $< 1.4 \times 10^{-15}$, with standard deviation between 0.020 and 0.073.

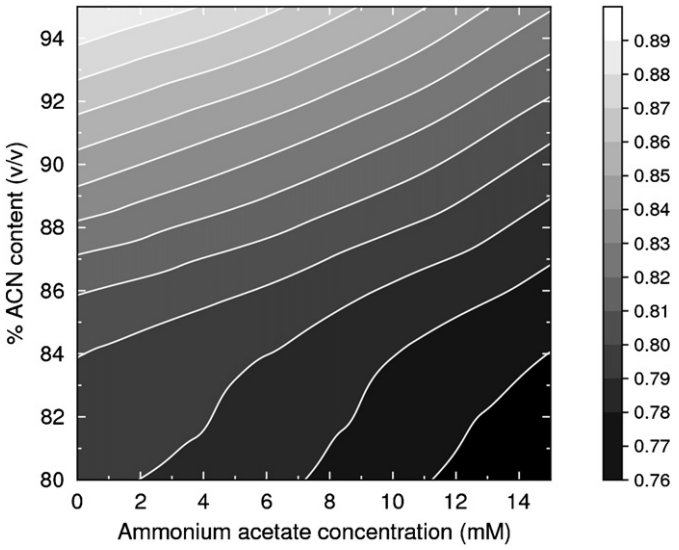

Fig. 5. Contour plot of toluene retention time (min) vs. the salt concentration (mM) and the % ACN content (v/v) in the mobile phase. Mobile phase: ACN/water/ammonium acetate at various concentrations; pH 7.0; column temperature, 20 °C; flow rate, 0.4 mL/min.

4. Conclusion

In the present study, the retention mechanism of a sulfobetaine phase in HILIC was investigated through the analysis of the retention behavior of hydroxy- and aminobenzoic acids. The effect of the chromatographic condition variation on their retention was carefully examined and related to their structural features. In particular, benzoic acids retention increased with increasing number and hydrogen donor ability of the polar functional groups, unless these interact with the carboxylate group. Several retention models were applied in order to investigate the contribution of electrostatic interactions, partitioning, and adsorption processes in the retention mechanism. Specific conclusions about the sulfobetaine phase of ZIC-HILIC column have been drawn:

- The phase behaves as a negative charged stationary phase, influenced by both salt concentration and pH of the mobile phase. The salt buffer has an important effect in the suppression of electrostatic repulsion with the anionic benzoic acids. On the other hand, the pH of the mobile phase has influence only on the underivatized silanol groups, as the sulfobetaine ligands are not affected by pH variation. A decrease of electrostatic repulsion with negative charged compounds occurs decreasing the pH.
- The sulfobetaine phase retains water strongly, allowing the creation of a water enriched layer on its surface even at high ACN content in the mobile phase (80–95%, v/v). As consequence, the retention behavior of the benzoic acids is well described by partition-like models. Due to presence of the H-acceptor sulfonate group in the sulfobetaine ligand, also adsorption-like processes contribute to the retention of analytes with H-donor functionalities.
- Both ACN and salt content in the mobile phase have been proved to affect the thickness of the water layer on the surface of the stationary phase.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2012.02.031.

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